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Macrophages and Multicellular Tumor Spheroids in Co-Culture: A Three-Dimensional Model to Study Tumor-Host Interactions

Evidence for Macrophage-Mediated Tumor Cell Proliferation and Migration

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In a new co-culture model involving multicellular tumor spheroids and different phenotypes of human macrophages, we studied the effects of the latter on migration and proliferation of the human colon carcinoma cell line, HRT-18. The macrophage phenotypes are detectable with monoclonal antibodies and are inducible in culture. 12-O-tetradecanoyl-phorbol-13-acetate-activated macrophages are associated with the phenotype 27E10, which is an acute inflammatory macrophage. The glucocorticoid-induced macrophage phenotype RM3/1 is associated with the down-regulation of inflammation. The phenotype resembling the mature resident macrophage termed 25F9 arises spontaneously in prolonged culture. It could be shown that inflammatory macrophages are localized at invasive areas of the tumor-host interface of colorectal carcinoma, whereas resident and anti-inflammatory macrophages were found in the central tumor region or at well-bordered areas of the tumor-host interface. The results obtained with this co-culture model show that 27E10-associated macrophages stimulate tumor cell migration and inhibit tumor cell proliferation. RM3/1 had only a slight inhibiting effect on proliferation and a slight promoting effect on migration. The 25F9-positive macrophage-stimulated tumor cell proliferation and inhibited migration completely. This investigation indicates that this in vitro system is useful for studying different macrophage effects on tumor cells and that indeed proliferation and migration of tumor cells could be influenced in an opposite manner by different types of macrophages. (Am J Pathol 1993, 143:1406–1415)

The presence of macrophages in malignant tumors, sometimes in large numbers, has been well-documented, but the significance of this phenomenon is still an open question.[1] Research in the field of interaction between macrophages and tumor cells deals in most instances with their cytotoxic properties. However, there is a growing body of evidence that tumor-associated macrophages play a key role in the promotion of tumor growth. The heterogeneity of the mononuclear phagocytic system is well-known and must be taken into account when assessing their activity in relation to tumor cells. The various phenotypes may have different, sometimes diametrically opposite, effects on the behavior of tumor cells. Recently, three phenotypically and functionally well-characterized types of human macrophages, recognized by the monoclonal antibodies 27E10, RM3/1, and 25F9 were reported.[2–6] Their behavior in suspension cultures is well-documented.[2–6] *In situ*, the 27E10-positive phenotype represents an inflamma-

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tory active macrophage,[2,6] whereas the glucocorticoid-inducible RM3/1-positive macrophage is associated with the down-regulation of inflammation.[3,5] The 25F9-positive phenotype is the adult resident macrophage[4] that seems to be associated with tumor progression.[7,8] Recently, we demonstrated that 27E10-positive macrophages showed a strong association with infiltrative areas of the tumor-host interface (THI) in colorectal carcinomas, whereas the 25F9-positive and the RM3/1-positive phenotype were found particularly in the stroma of the central tumor part and at well-bordered areas of the THI (manuscript in preparation). These observations suggest different functional activities of these macrophage phenotypes. Therefore, we were interested in a co-culture system using these different macrophages and human colorectal tumor cells. Here we present a culture system in which multicellular tumor spheroids of the human rectal carcinoma cell line HRT-18 were confronted with a suspension culture of human macrophages of distinct phenotypes on adherent (collagen type I) substratum to study tumor cell migration and on nonadherent (agarose) substratum for investigation of tumor cell proliferation. This model system allows lower numbers of macrophage-tumor cell contacts compared to the monolayer culture. Therefore, it more closely resembles the situation *in vivo*. The following data shows that different types of macrophages have different effects on tumor cells in this *in vitro* system.

Material and Methods

Monocytes

Monocytes were isolated from buffy coats (Blood Bank, Aachen, Germany) from individual donors with Ficoll-Paque (Pharmacia, Feiburg, Germany), followed by hypotonic density gradient centrifugation in Percoll (Pharmacia).[4] The cells were cultivated in hydrophobic Teflon bags in RPMI 1640 medium (Gibco, Laboratories Grand Island, NY) with 10% pooled human serum at a cell density of 2×10^6/ml. To induce the inflammatory 27E10 phenotype, monocytes were cultured for 2 days in the presence of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (10^{-9} mol/L)[2]; to stimulate the anti-inflammatory phenotype, RM3/1 monocytes were cultured with the glucocorticoid Prednylidene (10^{-7} mol/L) for 2 days.[5] To obtain mature macrophages of the phenotype 25F9, monocytes were cultured without stimulation for 7 days.[4] Under treatment with 12-O-tetradecanoyl-phorbol-13-acetate 20 to 40% of macrophages expressed the 27E10 antigen but only few (<10%) were positive for RM3/1-antibody. Glucocorticoid-treated cells revealed about 70 to 80% positive cells for RM3/1 but only 10% 27E10-positive macrophages. Monocytes cultured for 7 days express the 25F9-antigen in 50 to 70%, the 27E10 antigen in less than 10% and RM3/1 antigen in about 30%. 12-O-tetradecanoyl-phorbol-13-acetate-treated macrophages were therefore referred to as 27E10 cells, glucocorticoid-induced macrophages as RM3/1, and macrophages of day 7 of culture as 25F9.

Tumor Cells

The phenotypically heterogenous human rectum cancer cell line HRT-18,[9] which is able to produce multicellular tumor spheroids, was used between passage 92 and 102. Tumor cells were grown as monolayers in 25-cm^2 plastic tissue culture flasks in RPMI 1640, supplemented with 10% pooled human serum. To culture spheroids, cells were detached with 0.05% trypsin-ethylenediaminetetraacetic acid (Sigma Chemical Co., St. Louis, MO), washed once in medium, and plated on agarose-coated (1% agarose, Serva, Heidelberg, Germany) wells in a 96-microwell cluster plate (Greiner, Soluigeu, Germany) at a cell density of 1,000 cells/100 µl. Within 4 days, solid cell aggregates were formed.

Co-Culture

The co-culture was performed in a 24-well tissue culture plate. The wells were coated with 1% agarose (Serva) (anti-adherent) or collagen type I (Nitagel, Osaka, Japan) (adherence-promoting). One 4-day-old multicellular tumor spheroid (MCTS), containing approximately 1×10^4 cells was transferred to a well and covered with a suspension of macrophages (1×10^5). This results in a macrophage target ratio of 10:1. The co-culture duration was 5 days for each phenotype. Changes in the spheroid configuration were observed and documented daily by photomicroscopy. All experiments were performed in triplicate, that is, with macrophages from three donors. Within one set of experiments, three individual spheroids were used for migration assays and five spheroids for proliferation assays.

Immunocytochemistry

Cytospin preparations were made daily from two wells of co-culture with RM3/1 and 27E10. The stain-

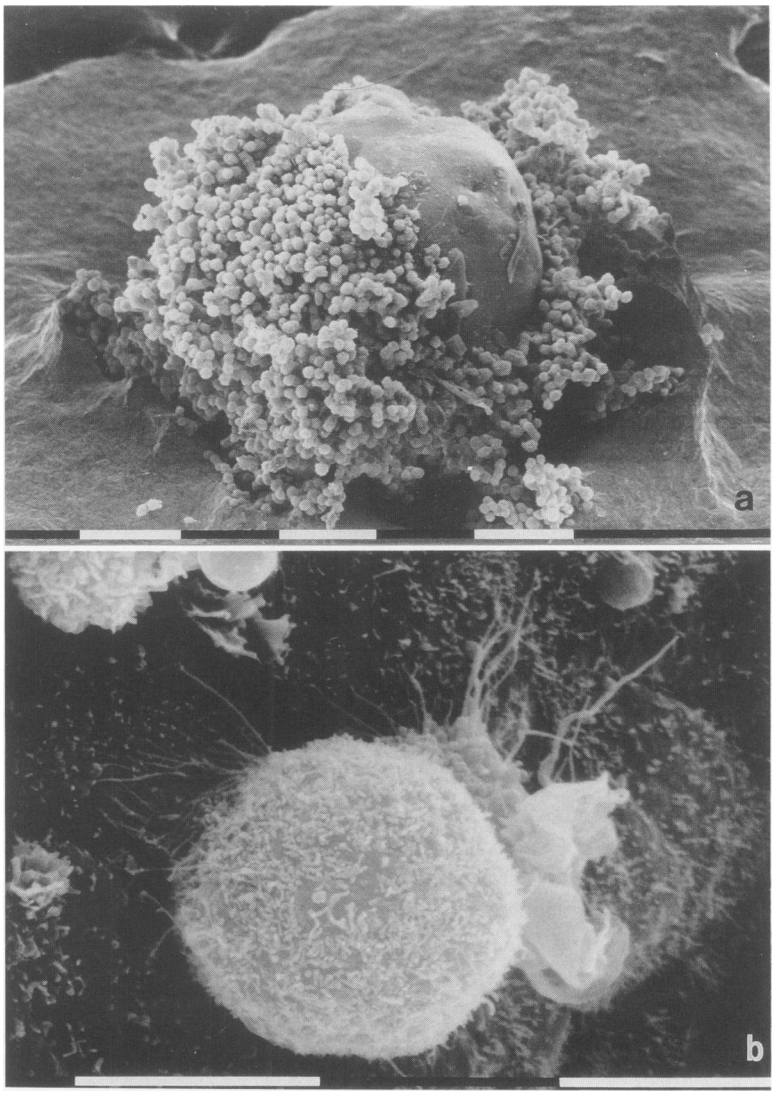

Figure 1. *Scanning electron microscopy of 27E10 co-culture after 24 hours.* a: *Abundant adhering macrophages on the surface of the MCTS. Scale bar = 100 μ.* b: *Filiform processes of macrophages in contact with the underlying tumor cells. Scale bar = 10 μ.*

ing was performed with the monoclonal antibodies 27E10, RM3/1, and 25F9 (Dianova, Hamburg, Germany) using an indirect immunofluorescence technique. This enabled quantitative assessment of the amount of either 27E10, RM3/1, or 25F9-positive macrophage.

Transmission and Scanning Electron Microscopy

In the early phase of the co-culture, spheroids remained as solid cell groups and could be handled for transmission electron microscopy as small tissue specimens. Disaggregated late-stage cultures were handled as cell suspensions. The contents of one well were transferred to an Eppendorf tube and centrifuged gently. The pellet was embedded in agarose, removed from the tube, and processed in the routine way for embedding in Epon. For scanning electron microscopy only, early-stage cultures were useful. The spheroids were removed from the well and dehydrated with increasing concentrations of ethanol. After critical point drying, the spheroids were gold-sputtered.

Migration Assay

One MCTS was placed in the center of a collagen-coated well (24-well plate) and overlayed with the macrophage suspension culture. A photomicrograph was made from the initial situation and repeated at 24-hour intervals. The slides were projected on a planimetry table. The area within the outer line of the MCTS or the migration halo was evaluated using a computer with image analyzing software. Migration was expressed as the increase

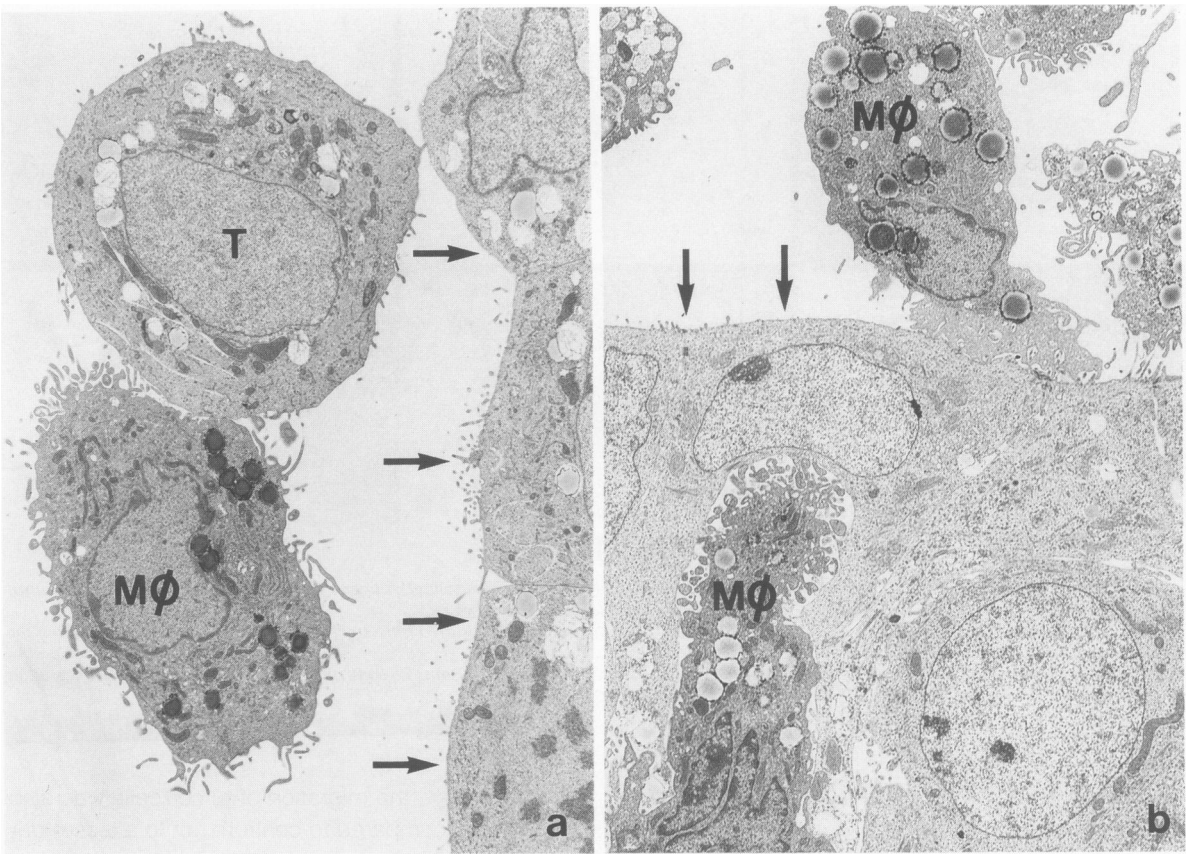

Figure 2. *Electron micrograph from a 24-hour co-culture with 27E10-positive macrophages.* **a:** *Detaching tumor cell- (T) macrophage (M) complexes are visible at the surface of the MCTS (arrows), magnification 5040×.* **b:** *One macrophage (M) is found within the spheroid, magnification 3060×.*

in area compared with the original area of the MCTS at the beginning of the experiment (time zero).

Proliferation Assay

This assay was performed under identical conditions as described for the migration assay except for the use of an agarose-coated substratum in all cases. At the beginning of co-culture and at 24-hour intervals, 0.5 μCi ^{3H}-thymidine (specific activity 5 to 20 Ci/mmol; Amersham, Braunschweig, Germany) was added to the well. After 3 hours, the content of the well was removed, washed for removal of nonincorporated thymidine, and lysed in 0.5 mol/L NaOH (Merc, Frankfurt, Germany). The amount of [^{3}H]thymidine incorporation was determined by liquid scintillation counting in a LKB-Wallac 1214 Rackbeta. The counts were expressed per mg protein, determined using the Bradford-reagent. All experiments were performed in triplicate.

Results

Agarose Cultures

Co-cultures of 27E10 macrophages and HRT-18 cells revealed that within the first 24 hours, macrophages became adherent to the surface of the MCTS, with long filiform processes present adjacent to the tumor cells (Figure 1, a and b). Sometimes they were also found within the MCTS. The intercellular contacts between the tumor cells in the periphery of the MCTS seemed to be less firm, because a detachment of macrophage-tumor cell complexes was often observed (Figure 2, a and b). On agarose, after 24 hours of co-culture, some small daughter-spheroids were observed (Figure 3a). After 5 days, these daughter spheroids were almost completely disintegrated (Figure 3b), suggesting an influence on spheroid formation in co-culture. The course of co-culture with RM3/1 was nearly the same as that observed with 27E10. The aggregates on agarose were, however, smaller but more numer-

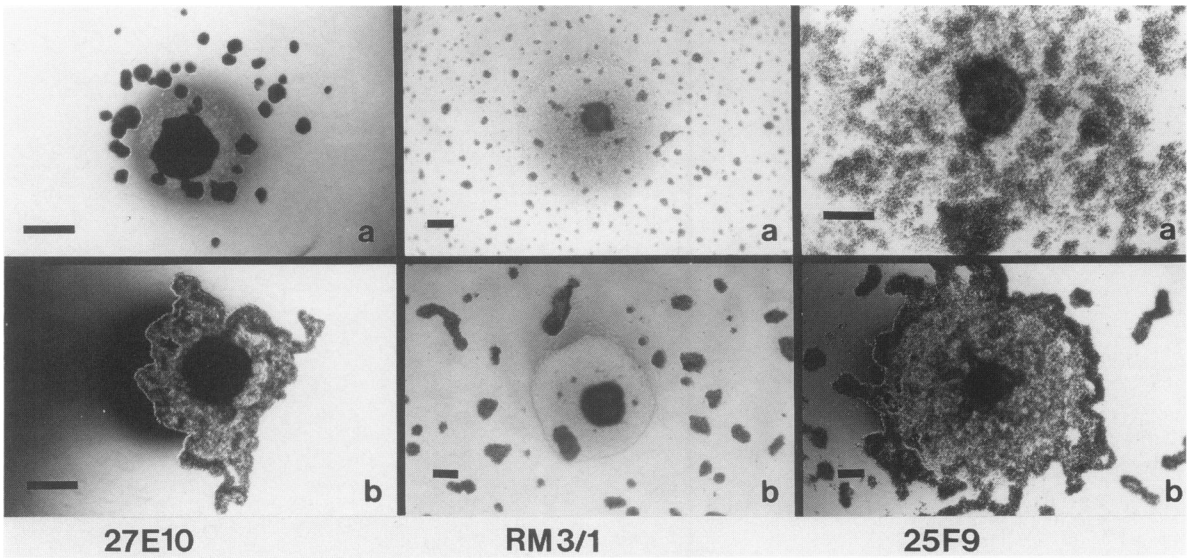

Figure 3. *Phase contrast micrograph of a co-culture with 27E10.* **a:** *After 24 hours, some small daughter spheroids are visible beside the central spheroid.* **b:** *After 69 hours, a marked disintegration of spheroids is visible. Scale bar = 500 μ.*
Figure 4. *Phase contrast micrograph of a co-culture with RM3/1.* **a:** *Many small spheroids are visible after 24 hours.* **b:** *After 69 hours, most of the small spheroids still persist. Scale bar = 500 μ.*
Figure 5. *Phase contrast micrograph of a co-culture with 25F9.* **a:** *Disintegration of MCTS starts already after 24 hours.* **b:** *Complete disintegration after 69 hours resembling the picture in the late-stage culture with 27E10. Scale bar = 500 μ.*

ous compared to 27E10 and some small aggregates were found until day 5 (Figure 4, a and b).

In co-cultures with 25F9, the tendency to dissolution was observed as early as 24 hours (Figure 5) and resembled the situation in the late phase of co-culture with 27E10. After 48 hours, most of the aggregates had undergone disintegration. This dissolution of MCTS was not the result of cytotoxicity, as shown by vital staining using trypan blue exclusion. Ultrastructurally, after 48 hours of co-culture, large cell cords were visible and consisted of tumor cells and macrophages. The tumor cells tended to be present in the center of these cords. Although they were closely packed, a distinct reduction of desmosomal structures was observed. Macrophages were present between the tumor cells and as an outer rim of the cords. No evidence of degenerative change in tumor cells was seen (Figure 6, a and b).

Migration Assay

The adhesive substrate collagen type I induced a spontaneous migration of tumor cells from the periphery of a MCTS. This migration was markedly influenced by co-culture with macrophages (Figure 7). In the presence of 25F9 macrophages, the tumor cells showed no migration. Co-culture with 27E10 macrophages resulted in an enhancement of migration compared to control cultures on collagen I. With RM3/1, the migration of tumor cells was also enhanced (compared to control), but to a lesser degree than 27E10.

Proliferation Assay

25F9-positive macrophages, as shown in Figure 8, are capable of stimulating tumor cell proliferation. This was particularly marked on day 2 of co-culture with an increase of counts over the control of 36.9%. RM3/1 caused a slight reduction of proliferation within the first 3 days of co-culture and reached the control value at day 4. 27E10-positive macrophages inhibited tumor cell proliferation to a marked degree within the first 48 hours of co-culture. Thereafter, proliferation reached the control level. The phenotypical instability of the 27E10 phenotype was studied using quantitative immunocytochemistry. As demonstrated in Figure 9, 27E10-positive macrophages decreased with time within the original 27E10-MCTS co-culture and 25F9-positive macrophages increased. The macrophages showed no proliferation.

Discussion

In the co-culture system described here, tumor cells are present as MCTS allowing the contact to macrophages only at the surface of the spheroids. This re-

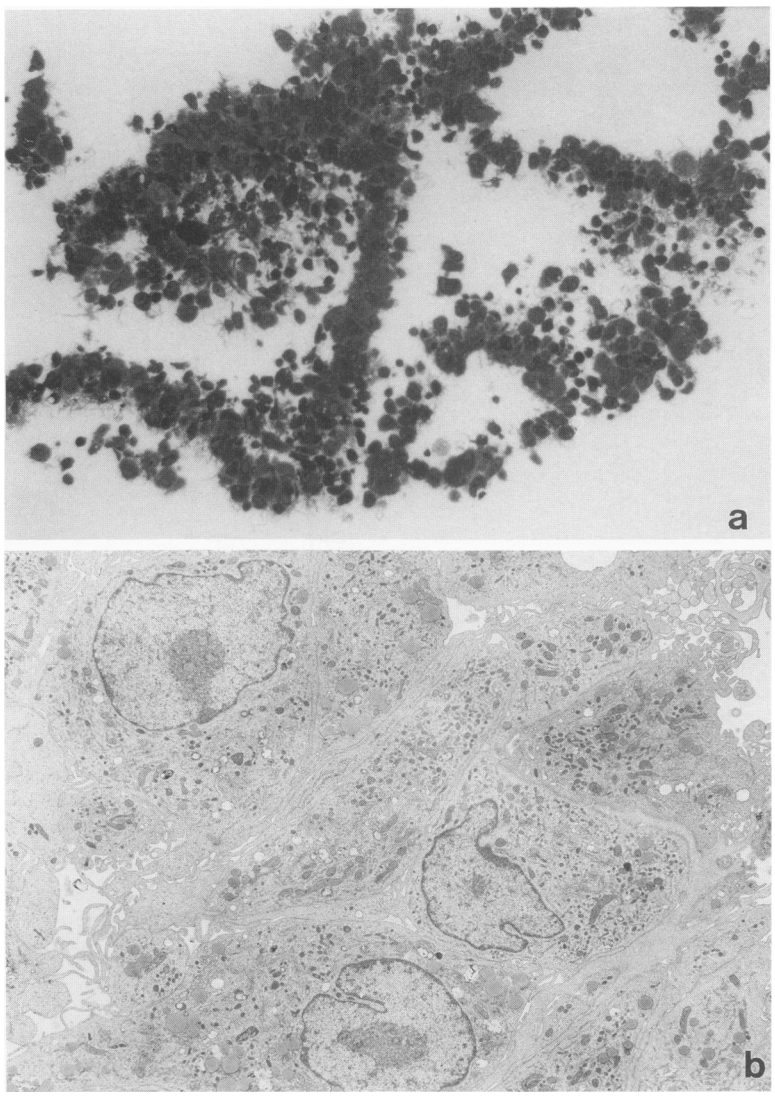

Figure 6. a: *Inhibition of MCTS-formation in 48 hours of co-culture with 25F9 with trabecular arrangement of tumor cells. Macrophages are seldom interposed between tumor cells and lie at the surface of trabeculae (semi-thin, 400×).* b: *Electron micrograph shows a marked reduction of desmosomes within the trabeculae, magnification 18,000×.*

sembles the situation *in vivo*, where tumor-associated macrophages were found predominantly at the tumor-host interface and only seldom interepithelially. Furthermore, the culture conditions used prevent the adherence of macrophages to the plastic bottom of the well. This is necessary because adherence to plastic surfaces induces marked changes of the functional properties of macrophages,[10,11] eg, the release of TxB_2 and LTB_4[12] and the induction of cytotoxicity.[13] We believe that our co-culture system preserves the characteristics of macrophages and takes into account their topographical distribution *in situ*. Additionally, the effects of macrophages on tumor cells also depend on the effector target ratio. The content of macrophages within malignant tumors was described as ranging between 10 and 60%.[14–17] We employed an effector target ratio of 10:1 with the intention that 5 to 10% of the macrophages will be in contact with the MCTS resulting in a definitive ratio of 1:1 to 1:2.

In our experiments, we used human macrophages of different phenotypes obtained by different culture treatments. Even though the macrophage cultures used contain no pure macrophage subtypes, the different behavior of tumor cells suggests that the induced macrophage phenotypes were responsible for these effects. First preliminary results of current studies with highly purified antibody positive for macrophage subsets (98%) showed the same effects (data not shown).

The mature, 25F9-positive macrophage was reported to be associated with high-stage malignant melanoma[7] and gastric carcinoma.[8] We found this macrophage in the central regions of colorectal carcinomas and at well-bordered areas of the THI. In co-culture, the proliferation of tumor cells was stimu-

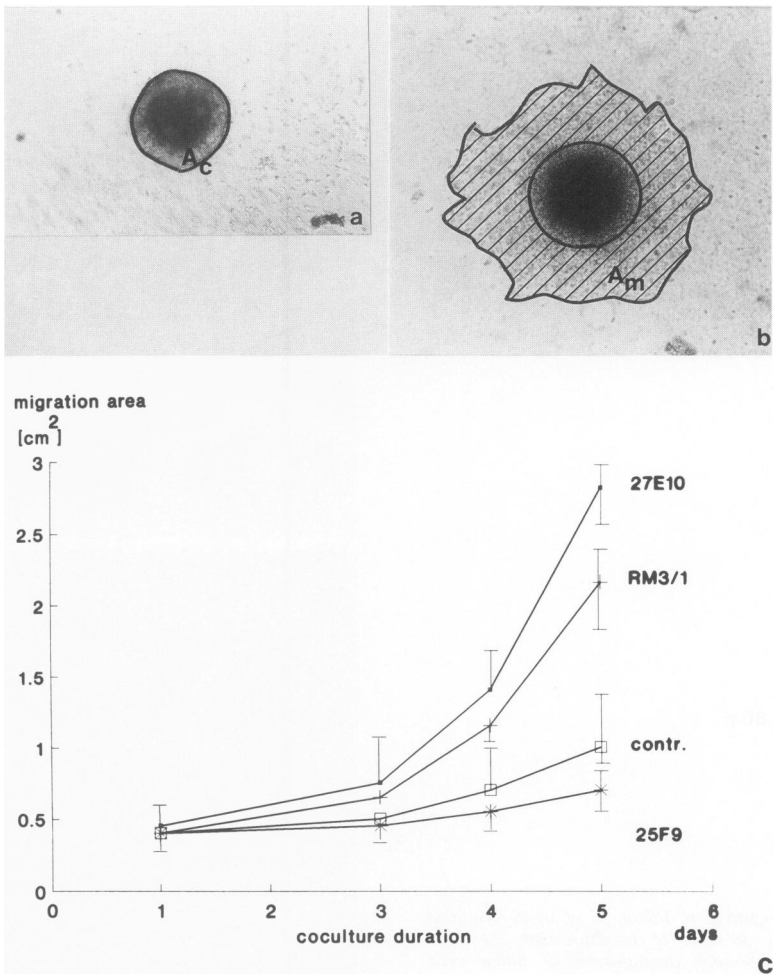

Figure 7. *Migration of tumor cells in co-culture on collagen type I with different macrophage phenotypes (effector target ratio 10:1).* a: *The initial situation shows a MCTS with sharp boundaries. Ac marks the area of the spheroid immediately after introducing into the well.* b: *After 48 hours, a marked migration halo is visible characterized by the area Am.* c: *Influence of different macrophage phenotypes on the migratory behavior of tumor cells.*

lated by this phenotype over the whole co-culture period. The enhancement of tumor growth and augmentation of metastasis by macrophages has been described by many authors.[18–21] The factors responsible for the stimulation of proliferation have not been characterized so far. Studies on the secretory function of this phenotype revealed no detectable amounts of tumor necrosis factor-α or interleukin-1 (unpublished results).

The macrophage 27E10 is a typical inflammatory macrophage producing large amounts of tumor necrosis factor-α, prostaglandin E_2 and interleukin-1.[2,6] This subtype was found in high numbers at invasive areas of the THI of colorectal carcinoma. As expected and indicated by a number of studies, inflammatory macrophages inhibited tumor cell proliferation.[18,22–25] However, after 48 hours, a marked increase in the proliferating activity of tumor cells was observed, indicating that the state of activation for tumor cell cytostatis is transient or requires activation.[26–29] Our immunocytochemical studies indicate that the maturation of 27E10-positive macrophages into the mature, 25F9-positive phenotype, which leads to a stimulation of tumor cell proliferation, is probably responsible for this phenomenon. Another explanation could be that the sensitive tumor cells will be killed and the resistent cells proliferate.

The RM3/1-positive macrophage represents an anti-inflammatory phenotype that is glucocorticoid-inducible.[3,5] Our results indicate that this subtype had no significant effects on migration or proliferation of tumor cells. The mechanisms involved in producing numerous small daughter spheroids remain unclear.

Besides the ability of macrophages to alter the proliferation of tumor cells, there is no doubt that they play a significant role in tumor invasion, eg, by degradation of extracellular matrix by enzymes released from macrophages.[30] We found a stimulation of tumor cell migration by the inflammatory macrophage 27E10. This is in agreement with our

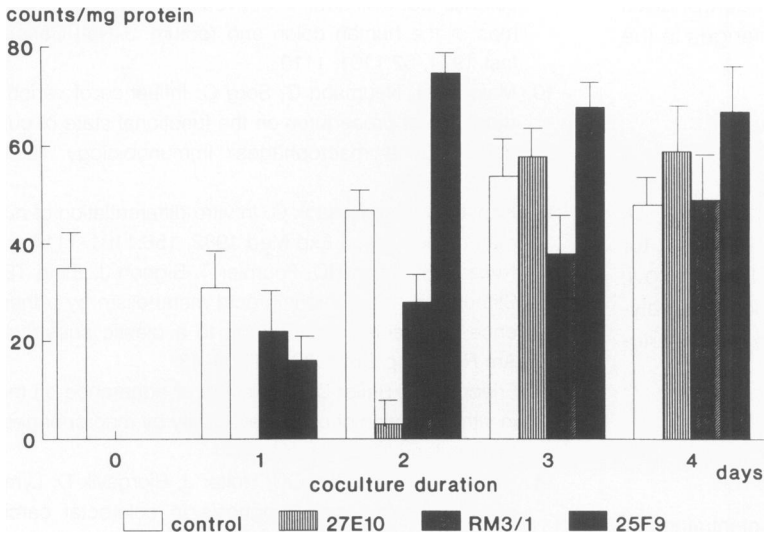

Figure 8. *Thymidine incorporation per mg protein of tumor cells in co-culture on agarose with different macrophage phenotypes (effector target ratio 10:1). Macrophages showed no thymidine incorporation (data not shown).*

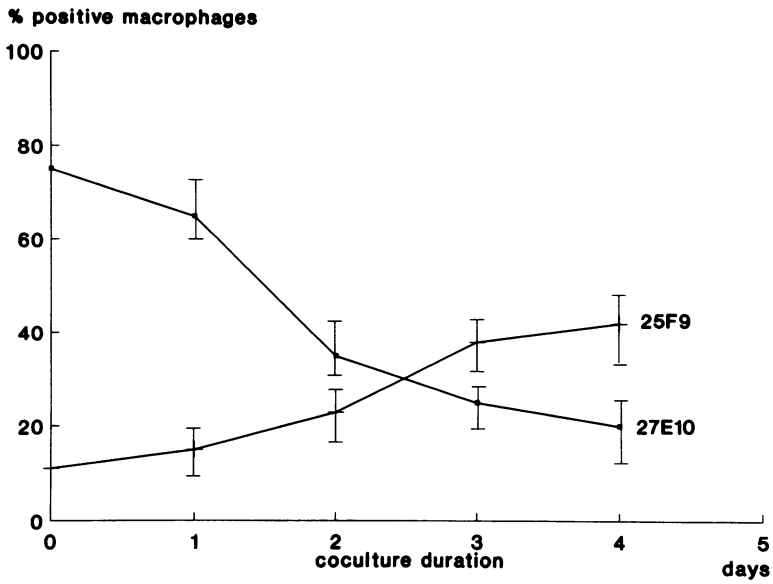

Figure 9. *Phenotypical shift of 27E10 macrophages in co-culture with tumor cells on agarose. The percentage of 27E10-positive macrophages decreases as a result of maturation into the 25F9-positive macrophage.*

light microscopical observation that this phenotype occurs predominantly at invasive areas of colorectal carcinomas. It is not clear which mediator is responsible for this phenomenon. However, investigations by Tamm et al[31] indicate that interleukin-6 acts as a scatter factor in a breast cancer cell line and may thus stimulate migration. On the other hand, tumor cell migration was inhibited in co-culture with 25F9-positive macrophages, which, as described above, stimulate tumor cell proliferation, indicating an inverse relationship between proliferation and migration. Invasion requires the loosening of the glandular structure of the carcinoma resulting in single cells, which are able to migrate. In late-stage cultures of 27E10 and 25F9, the MCTSs were completely disintegrated. By transmission electron microscopy, it could be shown that a reduction of desmosomes occurred that could be one reason for this effect. The behavior of cellular adhesion molecules and integrins in the co-culture are currently under study. Furthermore, many small cellular aggregates were found in the periphery of the introduced spheroid, a phenomenon that requires further investigation.

In conclusion, our results show that the MCTS-macrophage co-culture system provides a useful model to study the interaction between tumor cells and different macrophage subpopulations. Further studies are necessary to analyze the tumor-mediated

alteration of the secretory functions of macrophages and the mechanisms responsible for changes in the migratory behavior.

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